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Growth and activities of enzymes of primary metabolism in batch cultures of *Catharanthus roseus* cell suspension under different pCO_2 conditions

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Abstract. In vitro enzyme activities of glycolysis, pentose-phosphate pathway and dark CO_2 fixation were assayed in batch cultures of heterotrophic *Catharanthus roseus* cells under various gassing rates and partial pressures of carbon dioxide. Detrimental effects of low pCO_2 culture conditions on the growth characteristics could be linked to marked changes in levels of enzymes of primary metabolism during growth. The enzyme levels observed during the early stages of growth were found to be more stable when a constant pCO_2 (20 mbar) was maintained and enabled exponential growth to be reached more rapidly.

The importance of carbon dioxide as a "conditioning factor" of the culture medium is discussed.

Introduction

The development of plant cell culture technology has revealed gaseous transfer problems for large-scale cultivation in bioreactors. Some studies have suggested that in heterotrophic cell cultures, growth limitations might occur at low aeration rates due to oxygen starvation, while high aeration rates might be detrimental to growth as a consequence of a possible "stripping off" of key volatiles [7, 8, 13, 17, 18]. Otherwise, it has been shown that exogenous carbon dioxide is required for the growth of plant cells suspensions initiated at low cell densities [6, 12].

Previous results reported enhanced growth of *Catharanthus roseus* cells when Erlenmeyer flask cultures were performed in a CO_2-enriched environment [10]. The growth promoting effect of additional carbon dioxide could be explained by a stimulation of non-autotrophic CO_2 fixation, involving an enhanced substrate level for phosphoenol pyruvate carboxylase. As suggested by Schnabl & Mayer [14], this effect might be assigned to the enhanced operation of the Kreb's cycle as a result of a more rapid replenishment of

intermediates. In addition, the contribution of fixed CO_2 into biomass was calculated to be only 2% of the dry cell matter [11], supporting this hypothesis.

In a recent paper we reported that increasing the aeration rate from 0.4 to 1.5 volume of air per volume of liquid per minute led to a decrease of both global growth rate and conversion yield during large-scale cultivation of *Catharanthus roseus* cell suspensions in a 10 l bioreactor. In addition, no detrimental effect on the growth characteristics was observed upon increasing the gassing rate when the partial pressure of carbon dioxide in the culture was maintained at a constant level of 20 mbar using CO_2-enriched air as the sparging gas [2]. We concluded that carbon dioxide exerts a growth promoting effect during the early period of growth and that the pCO_2 needs to be considered as an operating parameter to ensure optimal growth in large-scale cultures.

In a recent review, Bown [1] examined the evidence for the intracellular pH modification in response to variations in pCO_2 levels and possible consequences, and suggested that CO_2-induced perturbation of intracellular pH may well influence transport processes at the plasma membrane, enzyme activities and intracellular concentration of hormones. However, the author concluded that given the meagre data available, speculation concerning the specific effects of CO_2 levels on metabolism was not warranted.

The aim of the present work was to provide information concerning possible linkage between changes in the capacity of enzymes of the primary metabolic pathways and previously observed modifications in growth characteristics of the cultures under various gaseous environments. To this end, in vitro activities of key enzymes of glycolysis, pentose phosphate pathway and dark CO_2 fixation were examined during growth under low and high pCO_2 levels.

Materials and methods

Cell suspension cultures

Catharanthus roseus (L.) G. Don cells were grown in 250 ml Erlenmeyer flasks containing 100 ml of culture medium described by Gamborg et al. [5], supplemented with 4.5 μM 2,4-dichlorophenoxyacetic acid and 0.28 μM kinetin, and buffered with MES (2-(N-morpholino) ethane sulfonic acid) 0.05 M, pH 6.0. Glucose was the sole carbon source (20 $g.l^{-1}$) and the initial biomass concentration was about 1 g d.w./l. Cell suspensions were cultivated on a rotary shaker (120 rpm) at 27 °C in the dark.

Culture systems

Erlenmeyer flask experiments were carried out in a CO_2 incubator (Forma Scientific model 3157) at the above mentioned shaking speed and temperature.

A stirred tank fermentor (Setric, France, Model SET 10 CM) with a working volume of 10 l was used for larger scale culture experiments. The carbon dioxide partial pressure in the culture medium was measured with a pCO_2 probe and an Ingold CO_2 amplifier. When necessary, the pCO_2 was maintained at a constant level (20 mbar) by supplying carbon dioxide to the inlet air flow.

Analytical methods

Growth rates were based on dry-weight measurements of the cell suspensions. Enzyme extraction and determination of enzyme activities were made by the following method. Cells were harvested by filtering 10 ml of culture through 8 μm pore size filters, washed with 10 ml buffer (0.1 M Na-phosphate pH 7.5, 5×10^{-4} M EDTA, 5×10^{-3} M mercaptoethanol), resuspended in the same volume of ice-cold buffer and disrupted for 30 s on ice using a Branson sonicator; completeness of breakage was monitored by microscopic observations. The homogenates were centrifuged at 8000 g for 5 minutes and the supernatants used for enzyme activity measurements. The enzymes investigated were hexokinase, HK (EC 2.7.1.1, glycolytic and pentose phosphate pathways); phosphofructokinase, PPFK (EC 2.7.1.11, glycolysis); glucose-6-phosphate dehydrogenase, G6PDH (EC 1.1.1.49, pentose phosphate pathway); 6-phosphogluconate dehydrogenase, 6PGDH (EC 1.1.1.44, pentose phosphate pathway); phosphoenolpyruvate carboxylase, PEPC (EC 4.1.1.31, dark fixation). Activities were assayed by following the oxidation or reduction of $NAD(P)^+$ or NAD(P)H at 340 nm and 30 °C using previously given protocols: HK [4], PPFK [3], G6PDH and 6PGDH [15, 16] and PEPC [11]. Reaction conditions were optimized for each enzyme as regards pH, substrate concentration and protein level to ensure that linear reaction kinetics as near maximum as possible were being measured. Assays represent the average of at least three determinations and repeated growth studies gave results of a similar nature. The protein contents of crude extracts were measured by the method of Lowry et al. [9] and were in the range of 9–12 g/100 g of cell dry weight. Enzyme activities were expressed as NKATAL per mg of cell protein.

Results and discussion

Enzyme activities during growth in Erlenmeyer flasks

The growth rate and the specific activities of enzymes during growth of *Catharanthus roseus* cells cultured in Erlenmeyer flasks under normal atmosphere are shown in Fig. 1. Maximum growth rate, duration of exponential phase and global conversion yield were $0.020\,h^{-1}$, 60% of the total culture time and $0.53\,g.g^{-1}$, respectively. The specific activities of all five enzymes fell during the early stage of the culture from levels representative of the exponential phase inoculum. During the period of rapid growth (acceleration and exponential phases) these activities regained their initial levels and in the case of HK, PPFK, G6PDH and 6PGDH diminished towards the end of exponential growth coincident with lowered sugar substrate levels. This variation in specific activity was most marked for HK, whose activity at all times was the lowest of any of the enzymes examined. The enzyme PEPC followed this general pattern but continued to increase until the end of the culture period, perhaps indicating a shift in metabolism towards enhanced CO_2-fixation at low sugar levels. The variation in pCO_2 levels presented in Fig. 1B agrees with this interpretation.

Small changes in the growth characteristics and in the profiles and the levels of enzyme activities occurred when similar experiments were undertaken within an incubator having a 20 mbar CO_2-enriched gas phase. The total growth period was shortened from 180 to 160 h and the global conversion yield increased up to $0.58\,g.g^{-1}$. As regards enzyme activities, the growth at higher CO_2 level showed a somewhat smaller fall in HK activity during early stage of growth and a higher PEPC activity. These slight differences were attributed to the rapid accumulation of respiratory carbon dioxide in the cultures of both series. Thus, in Erlenmeyer flasks the difference between air-phase and CO_2-enriched gas phase cultures was not necessarily representative of the profiles which might be expected in large-scale cultivations with constant air supply.

Enzyme activities during growth of large-scale cultures

To ascertain the effect of the carbon dioxide partial pressure on metabolic activity, growth parameters and enzyme activities were measured in large-scale bath cultures with a constant aeration rate. Three rates of aeration (0.4, 1.0 and 1.5 v.v.m.) were tried and a gassing rate of 1.5 v.v.m. retained in order to accentuate the effect of CO_2-stripping. With a normal air supply the pCO_2 never exceeded 0.3 mbar i.e. respiratory CO_2 was removed rapidly.

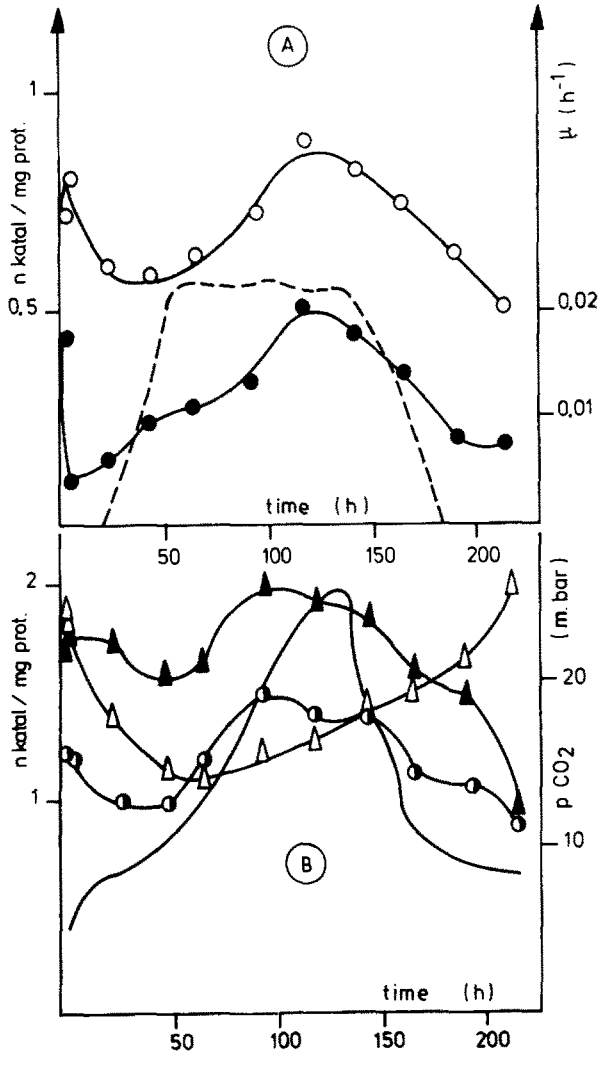

Fig. 1. Changes in specific activities of enzymes involved in carbohydrate metabolism during growth of *Catharanthus roseus* cells in Erlenmeyer flasks. A: ---- growth rate μ; ● HK; ○ PPFK. B: —— pCO_2; ◑ G6PDH; ▲ 6PGDH; △ PEPC.

The results obtained with such a system (Fig. 2) were compared with batch cultivations in which the pCO_2 was maintained at 20 mbar using CO_2-enriched air (Fig. 3).

The growth characteristics observed for cultures in which only a 0.3 mbar pCO_2 was reached were different from those where a 20 mbar pCO_2 was maintained. Although the maximum specific growth rate was the same, i.e.

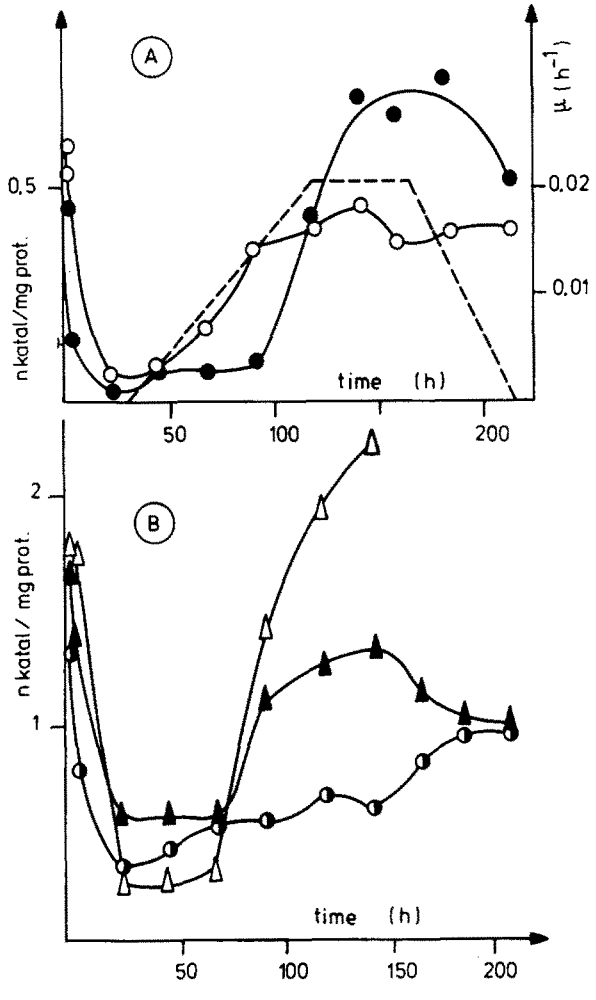

Fig. 2. Changes in specific activities of enzymes involved in carbohydrate metabolism during growth of *Catharanthus roseus* cells in a 10 l fermentor; aeration rate 1.5 v.v.m., normal air. Symbols as in Fig. 1.

$0.02\,h^{-1}$, for each series of experiments, those cultures with normal air showed a delayed onset of growth and a considerably extended acceleration phase followed by a shorter exponential phase (exponential growth phase only 18% of the total culture time for air-gased cultures, as compared to 58% for CO_2-enriched cultures). The global conversion yield of sugar to biomass was also better for CO_2-enriched culture, $0.53\,g.g^{-1}$ instead of $0.365\,g.g^{-1}$ in normal air-gased culture. With lower aeration rates, which allowed some build-up of CO_2 in the normal air-gased cultures, the differen-

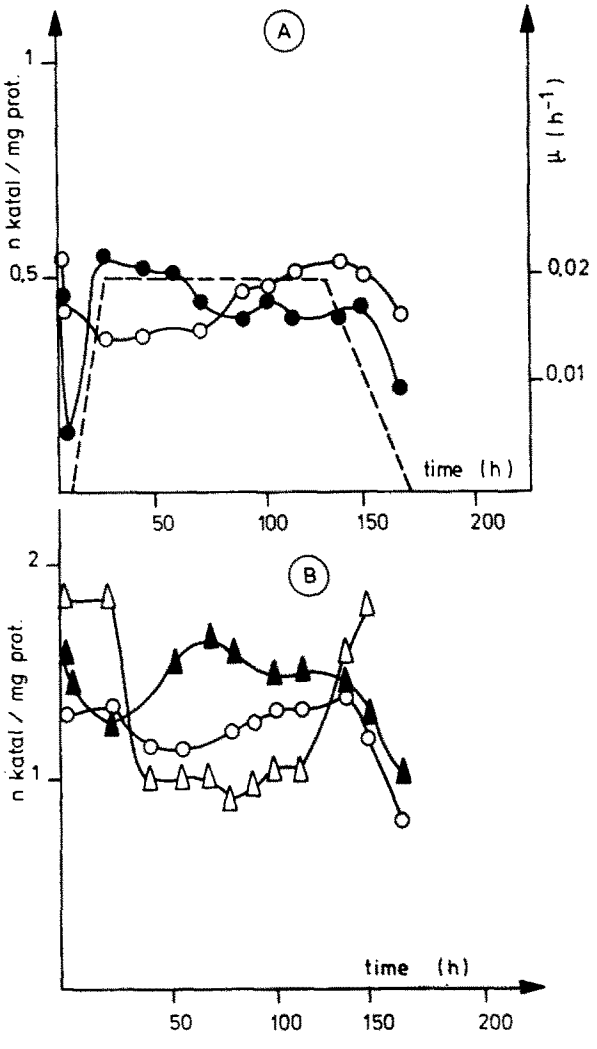

Fig. 3. Changes in specific activities of enzymes involved in carbohydrate metabolism during growth of *Catharanthus roseus* cells in a 10 l fermentor; aeration rate 1.5 v.v.m., CO_2-enriched air (pCO_2 20 mbar). Symbols as in Fig. 1.

ces in growth profile and in yield were less extreme (for detailed results of a similar study by these authors see Ref. 2).

The enzyme activities for air-gased cultures (Fig. 2) showed a similar profile to Erlenmeyer cultures but the initial fall in activity was greatly enhanced and these low levels persisted for a longer time. Hexokinase in particular did not show increased activity until the onset of exponential growth. When the cultures were grown under a 20 mbar pCO_2, the enzyme

profiles were somewhat different (Fig. 3). The variation in activity was considerably less obvious for PPFK and G6PDH until the end of exponential phase. HK and 6PGDH showed a more pronounced loss of activity at the beginning of the culture, this fall in enzyme levels being particularly marked for HK, but less extreme than when the cells were grown under normal air supply. PEPC showed a similar fall in activity which remained low throughout the exponential growth phase but increased sharply towards the end of this period.

In a subsequent experiment, we attempted to study the effects on the enzyme activities of both a decrease in the pCO_2 level and a dilution of the culture, and to compare them to those observed after the inoculation of a culture. During the first 40 hours the pCO_2 was maintained at 20 mbar following which the culture was sparged with normal air; after a further culture period of 100 hours, a 10-fold dilution of the culture was performed by replacing the culture broth by the same volume of fresh medium. As shown in Fig. 4, the gas shock imposed after 40 h of culture did not affect the growth nor the enzyme levels. In contrast a decrease of the specific activities of all the enzymes occurred as the result of the dilution of the biomass, but without modification of the growth rate. Dilution of a growing culture under normal air showed a similar response as regards enzyme levels to those observed following inoculation. Under high CO_2 gas atmospheres this fall in enzyme activity was largely restricted to HK. However, actively growing cells seemed better able to recover from dilution under low CO_2 atmospheres than was the case following inoculation.

It can be assumed that, with respect to primary metabolism, the disturbing effect following the transfer of cells into fresh medium is mainly related to the dilution of the culture. One has to consider that the inhibition of enzymes on subculturing the cells in a CO_2-deficient atmosphere could be linked to other factors including the gas shock, the age of inoculum, etc. . . During subcultivation, this negative effect on cell metabolism could be antagonized when the culture gas-phase was enriched with exogenous carbon dioxide.

In conclusion, the activities of some enzymes of carbohydrate metabolism, mainly hexokinase, were found to decline when heterotrophic cells were transferred into fresh medium. Our results were in close agreement with those of Fowler & Clifton [4] who concluded after examining the enzyme specific activities of *Acer pseudoplatanus* cells at different growth rates in continuous culture that these enzymes (particularly HK) were probably the major point of control for the flux of carbon through the various anabolic pathways. The enzyme profiles thus obtained from cells in a defined stage of growth were representative of the levels of enzymes

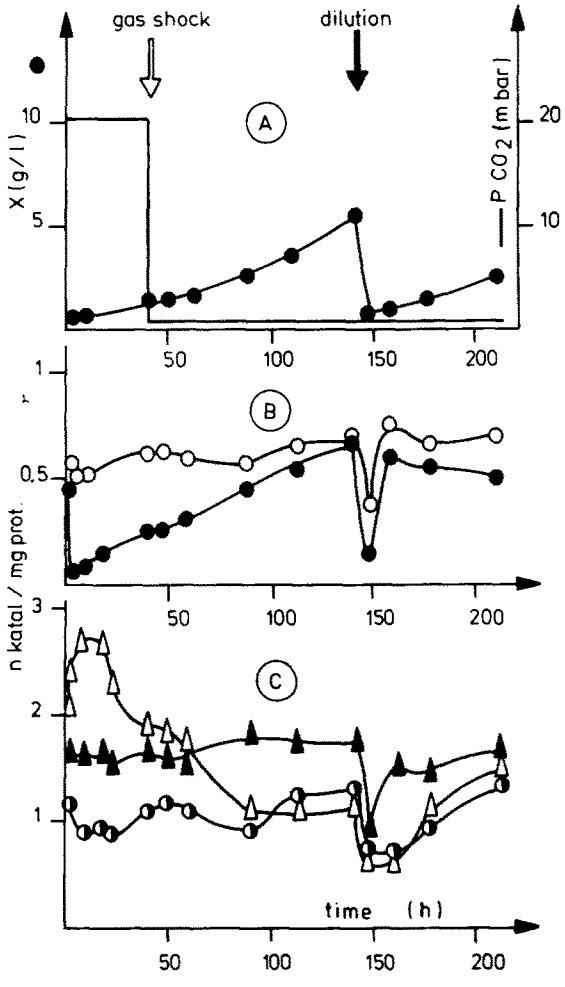

Fig. 4. Influence of gas shock or dilution of the suspension on enzyme activities of carbohydrate metabolism. A: ● biomass concentration; —— pCO_2. B and C: symbols as in Fig. 1.

observed at similar growth stages throughout a batch culture. Such enzyme activities were measured under in vitro conditions but should provide some indication of the catalytic capacities of primary metabolic pathways. As is the case for the growth rate, the observed changes in enzyme activities were found to be dependent on the cultural gaseous environment involving the partial pressure of carbon dioxide, at least in the early stages of growth.

Our observations underlined the fact that carbon dioxide, at low concentrations, may act as a "conditioning factor" of culture media for plant

cell suspensions. The partial pressure of CO_2 must be considered as an essential process variable for the optimized growth of plant cells in large-scale bioreactors which necessitate aeration.

In the future, the effect of pCO_2 should be subject to further investigation preferably in continuous culture so that the degree of enzyme variance can be fully quantified and related more exactly to the growth.

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